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On July 30, 2002

TOWNSEND and TOWNSEND and CREW LLP

By: [Signature]

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

LEPPLA *et al.*

Application No.: 10/088,952

Filed: March 22, 2002

For: MUTATED ANTHRAX TOXIN
PROTECTIVE ANTIGEN PROTEINS
THAT SPECIFICALLY TARGET
CELLS CONTAINING HIGH
AMOUNTS OF CELL-SURFACE
METALLOPROTEINASES OR
PLASMINOGEN ACTIVATOR
RECEPTORS

Examiner: Not yet assigned

Art Unit: Not yet assigned

COMMUNICATION UNDER

37 C.F.R. §§ 1.821-1.825

AND

PRELIMINARY AMENDMENT

U.S. Patent and Trademark Office
Box SEQUENCE
P.O. Box 2327
Arlington, VA 22202

Sir:

In response to the request to comply with Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures, 37 C.F.R. §§ 1.821-1.825, that accompanied the Notification of Missing Requirements Under 35 U.S.C. 351 in the United States Designated/Elected Office (DO/EO/US) mailed May 30, 2002, Applicants submit herewith the required paper copy and computer readable copy of the Sequence Listing. Please amend the specification in adherence with 37 C.F.R. §§ 1.821-1.825 as follows.

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In the Specification:

Please replace the paragraph beginning at page 1, line 17, with the following:

--Anthrax toxin is a three-part toxin secreted by *Bacillus anthracis* consisting of protective antigen (PA, 83 kDa), lethal factor (LF, 90 kDa) and edema factor (EF, 89 kDa) (Smith, H., *et al.*, *J. Gen. Microbiol.*, 29:517-521 (1962); Leppla, S.H., *Sourcebook of bacterial protein toxins*, p. 277-302 (1991); Leppla, S.H., *Handb. Nat. Toxins*, 8:543-572 (1995)), which are individually non-toxic. The mechanism by which individual toxin components interact to cause toxicity was recently reviewed (Leppla, S.H., *Handb. Nat. Toxins*, 8:543-572 (1995)). Protective antigen, recognized as central, receptor-binding component, binds to an unidentified receptor (Escuyer, V., *et al.*, *Infect. Immun.*, 59:3381-3386 (1991)) and is cleaved at the sequence RKKR₁₆₇ (SEQ ID NO:1) by cell-surface furin or furin-like proteases (Klimpel, K.R., *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:10277-10281 (1992); Molloy, S.S., *et al.*, *J. B. Chem.*, 267:16396-16402 (1992)) into two fragments: PA63, a 63 kDa C-terminal fragment, which remains receptor-bound; and PA20, a 20 kDa N-terminal fragment, which is released into the medium (Klimpel, K.R., *et al.*, *Mol. Microbiol.*, 13:1094-1100 (1994)). Dissociation of PA20 allows PA63 to form heptamer (Milne, J.C., *et al.*, *J. Biol. Chem.*, 269:20607-20612 (1994); Benson, E.L., *et al.*, *Biochemistry*, 37:3941-3948 (1998)) and also bind LF or EF (Leppla, S.H., *et al.*, *Bacterial protein toxins*, p. 111-112 (1988)). The resulting hetero-oligomeric complex is internalized by endocytosis (Gordon, V.M., *et al.*, *Infect. Immun.*, 56:1066-1069 (1988)), and acidification of the vesicle causes insertion of the PA63 heptamer into the endosomal membrane to produce a channel through which LF or EF translocate to the cytosol (Friedlander, A.M., *J. Biol. Chem.*, 261:7123-7126 (1986)), where LF and EF induce cytotoxic events.--

Please replace the paragraph beginning at page 2, line 31, with the following:

--The crystal structure of PA at 2.1 Å was solved by X-ray diffraction (PDB accession 1ACC) (Petosa, C., *et al.*, *Nature*, 385:833-838 (1997)). PA is a tall, flat molecule having four distinct domains that can be associated with functions previously defined by

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biochemical analysis. Domain 1 (aa 1-258) contains two tightly bound calcium ions, and a large flexible loop (aa 162-175) that includes the sequence RKKR₁₆₇ (SEQ ID NO:1), which is cleaved by furin during proteolytic activation. Domain 2 (aa 259-487) contains several very long β -strands and forms the core of the membrane-inserted channel. It is also has a large flexible loop (aa 303-319) implicated in membrane insertion. Domain 3 (aa 488-595) has no known function. Domain 4 (aa 596-735) is loosely associated with the other domains and is involved in receptor binding. For cleavage at RKKR₁₆₇ (SEQ ID NO:1) is absolutely required for the subsequent steps in toxin action, it would be of great interest to engineer it to the cleavage sequences of some disease-associated proteases, such as matrix metalloproteinases (MMPs) and proteases of the plasminogen activation system (e.g., t-PA, u-PA, etc., *see, e.g., Romer et al., APMIS* 107:120-127 (1999)), which are typically overexpressed in tumors.--

Please replace the paragraph beginning at page 6, line 25, with the following:

--In one embodiment, the cell overexpresses a matrix metalloproteinase. In another embodiment, the matrix metalloproteinase is selected from the group consisting of MMP-2 (gelatinase A), MMP-9 (gelatinase B) and membrane-type 1 MMP (MT1-MMP). In another embodiment, the matrix metalloproteinase-recognized cleavage site is selected from the group consisting of GPLGMLSQ (SEQ ID NO:2) and GPLGLWAQ (SEQ ID NO:3).--

Please replace the paragraph beginning at page 6, line 30, with the following:

--In one embodiment, the cell overexpresses a plasminogen activator or a plasminogen activator receptor. In another embodiment, the plasminogen activator is selected from the group consisting of t-PA (tissue-type plasminogen activator) and u-PA (urokinase-type plasminogen activator). In another embodiment, the plasminogen activator-recognized cleavage site is selected from the group consisting of PCPGRVVGG, PGSGRSA, PGSGKSA, and PQRGRSA (SEQ ID NOS:4-7, respectively).--

Please replace the paragraph beginning at page 7, line 28, with the following:

--In one embodiment, the matrix metalloproteinase or a plasminogen activator-recognized cleavage site is selected from the group consisting of PCPGRVVGG, PGSGRSA, PGSGKSA, PQRGRSA, GPLGMLSQ and GPLGLWAQ (SEQ ID NOS:4-7, 2 and 3, respectively).--

Please replace the paragraph beginning at page 7, line 33, with the following:

--Fig. 1. Generation of PA mutants can be specifically processed by MMPs. (A). Schematic representation of MMP substrate PA mutants. The furin cleavage site RKKR (SEQ ID NO:1) was replaced with gelatinase favorite substrate sequences GPLGMLSQ (SEQ ID NO:2) in PA-L1 and GPLGLWAQ (SEQ ID NO:3) in PA-L2. The arrows show the cleavage sites of furin or MMPs as indicated. (B). Cleavage of PA-L1 by MMP-2, MMP-9 and soluble form furin. As described in Materials and Methods, PA-L1 was incubated with MMP-2, MMP-9 and furin, respectively, aliquots were withdrawn at the time points indicated, and the samples were analyzed by western blotting with the rabbit polyclonal antibody against PA. (C). Cleavage of PA-L2 by MMP-2, MMP-9 and soluble form furin. PA-L2 was treated as in B. (D). Cleavage of WT-PA by MMP-2, MMP-9 and soluble form furin. WT-PA was treated as in B.--

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Please replace the paragraph beginning at page 19, line 26, with the following:

--Protective antigen proteins can be produced from nucleic acid constructs encoding mutants, in which the naturally occurring furin cleavage site has been replaced by an MMP or a plasminogen activator cleavage site. In addition, LF proteins, and LF and PA fusion proteins can also be expressed from nucleic acid constructs according to standard methodology. Those of skill in the art will recognize a wide variety of ways to introduce mutations into a nucleic acid encoding protective antigen or to construct a mutant protective antigen-encoding nucleic acid. Such methods are well known in the art (*see* Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2nd ed. 1989); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994)). In some embodiments, nucleic acids of the invention are generated using PCR (*see, e.g.*, Examples I and III). For example, using overlap PCR protective antigen encoding nucleic acids can be generated by substituting the nucleic acid subsequence that encodes the furin site with a nucleic acid subsequence that encodes a matrix metalloproteinase (MMP) site (e.g., GPLGMLSQ and GPLGLWAQ; SEQ ID NOS:2 and 3) (*see, e.g.*, Example I). Similarly, an overlap PCR method can be used to construct the protective antigen proteins in which the furin site is replaced by a plasminogen activator cleavage site (e.g., the uPA and tPA physiological substrate sequence PCPGRVVGG (SEQ ID NO:4), the uPA favorite sequence PGSGRSA (SEQ ID NO:5), the uPA favorite sequence PGSGKSA (SEQ ID NO:6), or the tPA favorite sequence PQRGRSA (SEQ ID NO:7)) (*see, e.g.*, Example III).--

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Please replace the paragraph beginning at page 34, line 26, with the following:

--Overlap PCR was used to construct the PA mutants with the furin site replaced by MMP substrate octapeptide GPLGMLSQ (SEQ ID NO:2) in PA-L1 and GPLGLWAQ (SEQ ID NO:3) in PA-L2. Wild type PA (WT-PA) expression plasmid pYS5 (Singh, Y., *et al.*, *J Biol Chem*, 264:19103-19107 (1989)) was used as template. We used 5' primer F (AAAGGAGAACGTATATGA (SEQ ID NO:8), underlined are SD sequence and start codon of PA) and the phosphorylated primer R1 (pTGAGTTCGAAGATTTTGTTTTAATTCTGG (SEQ ID NO:9), annealing to the sequence corresponding to P₁₅₄-S₁₆₃) to amplify the fragment N. We used the mutagenic phosphorylated primer H1 (pGGACCATTAGGAATGTGGAGTCAAAGTACAAGTGC TGGACCTACGGTTCCG (SEQ ID NO:10), encoding MMP substrate GPLGMLSQ (SEQ ID NO:2) and S₁₆₈-P₁₇₆) and reverse primer R2 ACGTTTATCTCTTATTAAAAT (SEQ ID NO:11), annealing to the sequence compassing I₅₈₉-R₅₉₅) to amplify the mutagenic fragment M1. We used a phosphorylated mutagenic primer H2 (pGGACCAT TAGGATTATGGGCACAAAGTACAAGTGCTGGACCTACGGTTCCG (SEQ ID NO:12), encoding MMP substrate GPLGLWAQ (SEQ ID NO:3) and S₁₆₈-P₁₇₆) to amplify mutagenic fragment M2. Then used primer F and R2 to amplify the ligation products of N and M1, N and M2, respectively, resulting in the mutagenic fragments L1 and L2, in which the coding sequence for furin site (RKKR₁₆₇; SEQ ID NO:1) were replaced by MMP substrate sequence GPLGMLSQ and GPLGLWAQ (SEQ ID NOS:2 and 3), respectively. The HindIII/PstI digests of L1 and L2, which included the mutation sites, were cloned between HindIII and PstI site of pYS5. The resulting expression plasmids were named pYS-PA-L1 and pYS-PA-L2, their expression products, the PA mutated proteins, were accordingly named PA-L1 and PA-L2.--

Please replace the paragraph beginning at page 39, line 20, with the following:

--Crystal structure of PA showed that the furin cleavage site RKKR₁₆₇ is in the middle of a surface flexible, solvent exposed loop composed of aa 162 to 175 (Petosa, C., *et al.*, *Nature*, 385:833-838 (1997)). Cleavage in this loop by furin-like proteases is essential to toxicity. To construct PA mutants specifically processed by MMPs, especially MMP-2 and MMP-9, instead of furin, the furin site RKKR₁₆₇ (SEQ ID NO:1) was replaced by MMP-2 and MMP-9 favorite sequences, GPLGMLSQ and GPLGLWAQ (SEQ ID NOS:2 and 3), respectively, resulting in two PA mutants, PA-L1 and PA-L2 (Fig. 1a). These two MMP substrate octapeptides were designed based on the studies of Netzel-Arnett *et al* (Netzel-Arnett, S., *et al.*, *J Biol Chem*, 266:6747-6755 (1991); Netzel-Arnett, S., *et al.*, *Biochemistry*, 32:6427-6432 (1993)), in which the sequence specificity of human MMP-2, MMP-9, matrilysin, MMP-1 and MMP-8 had been examined by measuring the rate of hydrolysis of over 50 synthetic oligopeptides. These two octapeptides are favorite substrates of MMP-2 and MMP-9, but also overlap to other MMP species (Netzel-Arnett, S., *et al.*, *J Biol Chem*, 266:6747-6755 (1991); Netzel-Arnett, S., *et al.*, *Biochemistry*, 32:6427-6432 (1993)). They are also potential substrates for MT1-MMP (Will, H., *et al.*, *J Biol Chem*, 271:17119-17123 (1996)). PA-L1 and PA-L2 coding sequences were constructed by overlap PCR, cloned into *E. coli*-*Bacillus* shuttle vector pYS5, and efficiently expressed in non-virulent *Bacillus Anthracis* UM23C1-1. The expression products were secreted into the culture supernatants and reached to 20 to 50 mg/L. These two mutated PA proteins were roughly purified by ammonium sulfate precipitation, followed by mono Q chromatography. The purified mutated PA proteins PA-L1 and PA-L2 commiserated with WT-PA in SDS-PAGE, but migrated faster than WT-PA in native gel because of the four positively charged residues RKKR (SEQ ID NO:1) of the furin site were replaced into non-charged MMP octapeptides (data not shown).--

Please replace the paragraph (Table 2) beginning at page 43, line 28, with the following:

--Table 2: u-TP and t-PA cleavage sites

Substrate sequence	SEQ ID NO:	u-PA Kcat/Km	t-PA Kcat/Km	a-PA:t-PA selectivity
PCPGRVVGG	4	0.88	0.29	3.0
PGSGRSA	5	1200	60	20
PGSGKSA	6	193	1.6	121
PQRGRSA	7	45	850	0.005

--

Please replace the paragraph beginning at page 44, line 16, with the following:

--A modified overlap PCR method was used to construct the mutated PA proteins in which the furin site is replaced by the uPA and tPA physiological substrate sequence PCPGRVVGG (SEQ ID NO:4) in PA-U1, uPA favorite sequences PGSGRSA (SEQ ID NO:5) and PGSGKSA (SEQ ID NO:6) in PA-U2 and PA-U3, respectively, tPA favorite sequence PQRGRSA (SEQ ID NO:7) in PA-U4. The PA expression plasmid pYS5 (Singh, Y., *et al.*, *J Biol Chem*, 264:19103-19107 (1989)) was used as template. A 5' primer F, AAAGGAGAACGTATATGA (SEQ ID NO:8) (Shine-Dalgarno and start codons are underlined), and the phosphorylated reverse primer R1, pTGGTGAGTTCTGA AGATTTTGTCTTAATTCTGG (SEQ ID NO:13) (the first three nucleotides encodes P, the others anneal to the sequence corresponding to P₁₅₄-S₁₆₃), were used to amplify a fragment designated "N". A mutagenic phosphorylated primer H1, pTGTCCAGGAAG AGTAGTTGGAGGAAGTACAAGTGCTGGACCTACGGTTCCAG (SEQ ID NO:14), encoding CPGRVVGG (SEQ ID NO:15) and S₁₆₈-P₁₇₆, and reverse primer R2, ACGTTTATCTCTTATTAAAT (SEQ ID NO:11), annealing to the sequence encoding I₅₈₉-R₅₉₅, were used to amplify a mutagenic fragment "M1". A phosphorylated mutagenic primer H2, pGGAAGTGGAAGATCAGCAAGTACAAGTGCTGGACCTAC GGTTCAG (SEQ ID

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NO:16), encoding GSGRSA (SEQ ID NO:17) and S₁₆₈-P₁₇₆, and reverse primer R2 were used to amplify a mutagenic fragment "M2". A phosphorylated mutagenic primer H3, pGGAAGTGGAAAATCAGCAAGTACAAGTGCTGGACCTA CGGTTCCAG (SEQ ID NO:18), encoding GSGKSA (SEQ ID NO:19) and S₁₆₈-P₁₇₆, and reverse primer R2, were used to amplify a mutagenic fragment "M3". A phosphorylated mutagenic primer H4, pCAGAGAGGAAGATCAGCAAGTACAAGTG CTGGACCTACGGTTCCAG (SEQ ID NO:20), encoding QRGRSA (SEQ ID NO:21) and S₁₆₈-P₁₇₆, and reverse primer R2, were used to amplify a mutagenic fragment "M4". Primers F and R2 were used to amplify the ligated products of N + M1, N + M2, N + M3, and N + M4, respectively, resulting in the mutagenized fragments U1, U2, U3, and U4 in which the coding sequence for the furin site (RKKR₁₆₇; SEQ ID NO:1) is replaced by uPA or tPA substrate. The *Hind*III/*Pst*I digests of U1, U2, U3, and U4 were cloned between the *Hind*III and *Pst*I sites of pYS5. The resulting expression plasmids were named pYS-PA-U1, pYS-PA-U2, pYS-PA-U3, and pYS-PA-U4, and their expression products, the mutated PA proteins, were accordingly named PA-U1, PA-U2, PA-U3, and PA-U4. One expression plasmid encoded a mutant in which RKKR₁₆₇ (SEQ ID NO:1) is replaced by PGG, expected not to be cleaved by any protease. Its expression plasmid and expression product were named pYS-PA-U7 and PA-U7, respectively.--

Please replace the paragraph beginning at page 48, line 6, with the following:

--The crystal structure of PA shows that the furin site, RKKR₁₆₇ (SEQ ID NO:1), is in a surface-exposed, flexible loop composed of aa 162 to 175 (Petosa, C., *et al.*, *Nature*, 385:833-838 (1997)). Cleavage in this loop by furin or furin-like proteases is essential to toxicity. Mutated PA proteins were constructed in which the furin-sensitive sequence RKKR₁₆₇ (SEQ ID NO:1) is replaced by uPA or tPA substrate sequences. In mutated PA protein PA-U1, PCPGRVVGG (SEQ ID NO:4), a peptide from P5 to P4' in the physiological substrate plasminogen, was used to replace RKKR₁₆₇ (SEQ ID NO:1). In PA-U2, RKKR₁₆₇ (SEQ ID NO:1) was replaced by a peptide, PGSGRSA (SEQ ID NO:5), containing the consensus sequence SGRSA (SEQ ID NO:22) from P3 to P2', which was recently identified as the

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minimized best substrate for uPA (Ke, S. H., *et al.*, *J. Biol. Chem.*, 272:20456-20462 (1997)). Because the peptide SGRSA (SEQ ID NO:22) is cleaved 1363-fold times more efficiently than a control peptide containing the physiological cleavage site present in plasminogen by uPA, and exhibits a uPA/tPA selectivity of 20 (Ke, S. H., *et al.*, *J. Biol. Chem.*, 272:20456-20462 (1997)), PA-U2 was expected to be a favorite substrate of uPA. uPA/tPA selectivity of the peptide SGRSA (SEQ ID NO:22) can be further enhanced by placement of lysine in the P1 position (Ke, S. H., *et al.*, *J. Biol. Chem.*, 272:20456-20462 (1997)), thus, the peptide PGSGKSA (SEQ ID NO:6), which exhibits a uPA/tPA selectivity of 121 (Ke, S. H., *et al.*, *J. Biol. Chem.*, 272:20456-20462 (1997)), was used to replace RKKR₁₆₇ (SEQ ID NO:1) to construct a mutated PA protein, PA-U3, with even higher uPA selective activity than PA-U2. The investigation showed P3 and P4 residues were the primary determinants of the ability of a substrate to discriminate between tPA and uPA, and mutation of both P4 glycine and P3 serine of the most labile uPA substrate (GSGRSA; SEQ ID NO:17) to glutamine and arginine, respectively, decreased the uPA/tPA selectivity by a factor of 1200 and actually converted the peptide into a tPA-selective substrate (Ke, S. H., *et al.*, *J. Biol. Chem.*, 272:20456-20462 (1997)). Based on this study, a mutated PA protein, PA-U4, was constructed. PA-U4 is expected to be a tPA favorite substrate, in which the peptide PQRGRSA (SEQ ID NO:7) was used to replace RKKR₁₆₇ (SEQ ID NO:1). A mutated PA protein PA-U7, was also constructed in which RKKR₁₆₇ (SEQ ID NO:1) was replaced by random sequence PGG, expected not to be cleaved by any known proteases, was used a control protein in this study. The designations of the mutated PA proteins along with the expected properties were summarized in Table 3.--

Please replace the paragraph (Table 3) beginning at page 55, line 1, with the following:

--Table 3. PA proteins generated in this study

Designation	Sequence at the "furin loop"		SEQ ID NO:	K _{cat} /K _m ¹		uPA:tPA selectivity ¹	Protease expected to cleave
				uPA	tPA		
PA	NS RKKR↑	STSAGPTV	23				Furin
PA-U1	NSPC <u>PGR</u> ↑ <u>VVGG</u> STSAGPTV		24	0.88	0.29	3	uPA/tPA (weakly)
PA-U2	NSPG <u>SGR</u> ↑ <u>SA</u>	STSAGPTV	25	1200	60	20	uPA
PA-U3	NSPG <u>SGK</u> ↑ <u>SA</u>	STSAGPTV	26	193	1.6	121	uPA
PA-U4	NSP <u>QRGR</u> ↑ <u>SA</u>	STSAGPTV	27	7.3	670	0.005	tPA
PA-U7	NSPGG	STSAGPTV	28				None

¹Data was cited from Ke, S. H., *et al.*, *J. Biol. Chem.*, 272:20456-20462 (1997) which was obtained from the studies on the peptides underlined in column 2.--

Please insert the accompanying paper copy of the Sequence Listing, page numbers 1 to 8, at the end of the application.

In the Claims:

Please amend claims 6, 7 and 24 as follows:

6. (Amended) The method of claim 1, wherein the matrix metalloproteinase-recognized cleavage site is selected from the group consisting of GPLGMLSQ (SEQ ID NO:2) and GPLGLWAQ (SEQ ID NO:3).

7. (Amended) The method of claim 1, wherein the plasminogen activator-recognized cleavage site is selected from the group consisting of PCPGRVVGG (SEQ ID NO:4), PGSGRSA (SEQ ID NO:5), PGSGKSA (SEQ ID NO:6), and PQRGRSA (SEQ ID NO:7).

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24. (Amended) The method of claim 23, wherein the matrix metalloproteinase or a plasminogen activator-recognized cleavage site is selected from the group consisting PCPGRVVGG (SEQ ID NO:4), PGSGRSA (SEQ ID NO:5), PGSGKSA (SEQ ID NO:6), PQRGRSA (SEQ ID NO:7), GPLGMLSQ (SEQ ID NO:2) and GPLGLWAQ (SEQ ID NO:3).

REMARKS

Applicants request entry of this amendment in adherence with 37 C.F.R. §§1.821 to 1.825. This amendment is accompanied by a floppy disk containing the above named sequences, SEQ ID NOS:1-28, in computer readable form, and a paper copy of the sequence information which has been printed from the floppy disk.

The information contained in the computer readable disk was prepared through the use of the software program "PatentIn" and is identical to that of the paper copy. This amendment contains no new matter.

Attached hereto is a marked-up version of the changes made to the Specification and Claims by the current Amendment. The attached pages are captioned "**VERSION WITH MARKINGS TO SHOW CHANGES MADE.**" As a convenience to the Examiner, a complete set of the Claims, as amended herein, is also attached to this Amendment as an Appendix entitled "**PENDING CLAIMS WITH ENTRY OF THE AMENDMENT.**"

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,



Annette S. Parent
Reg. No. 42,058

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

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Anthrax toxin is a three-part toxin secreted by *Bacillus anthracis* consisting of protective antigen (PA, 83 kDa), lethal factor (LF, 90 kDa) and edema factor (EF, 89 kDa) (Smith, H., *et al.*, *J. Gen. Microbiol.*, 29:517-521 (1962); Leppla, S.H., *Sourcebook of bacterial protein toxins*, p. 277-302 (1991); Leppla, S.H., *Handb. Nat. Toxins*, 8:543-572 (1995)), which are individually non-toxic. The mechanism by which individual toxin components interact to cause toxicity was recently reviewed (Leppla, S.H., *Handb. Nat. Toxins*, 8:543-572 (1995)). Protective antigen, recognized as central, receptor-binding component, binds to an unidentified receptor (Escuyer, V., *et al.*, *Infect. Immun.*, 59:3381-3386 (1991)) and is cleaved at the sequence RKKR₁₆₇ (SEQ ID NO:1) by cell-surface furin or furin-like proteases (Klimpel, K.R., *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:10277-10281 (1992); Molloy, S.S., *et al.*, *J. B. Chem.*, 267:16396-16402 (1992)) into two fragments: PA63, a 63 kDa C-terminal fragment, which remains receptor-bound; and PA20, a 20 kDa N-terminal fragment, which is released into the medium (Klimpel, K.R., *et al.*, *Mol. Microbiol.*, 13:1094-1100 (1994)). Dissociation of PA20 allows PA63 to form heptamer (Milne, J.C., *et al.*, *J. Biol. Chem.*, 269:20607-20612 (1994); Benson, E.L., *et al.*, *Biochemistry*, 37:3941-3948 (1998)) and also bind LF or EF (Leppla, S.H., *et al.*, *Bacterial protein toxins*, p. 111-112 (1988)). The resulting hetero-oligomeric complex is internalized by endocytosis (Gordon, V.M., *et al.*, *Infect. Immun.*, 56:1066-1069 (1988)), and acidification of the vesicle causes insertion of the PA63 heptamer into the endosomal membrane to produce a channel through which LF or EF translocate to the cytosol (Friedlander, A.M., *J. Biol. Chem.*, 261:7123-7126 (1986)), where LF and EF induce cytotoxic events.

Paragraph beginning at line 31 of page 2 has been amended as follows:

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Paragraph beginning at line 25 of page 6 has been amended as follows:

In one embodiment, the cell overexpresses a matrix metalloproteinase. In another embodiment, the matrix metalloproteinase is selected from the group consisting of MMP-2 (gelatinase A), MMP-9 (gelatinase B) and membrane-type 1 MMP (MT1-MMP). In another embodiment, the matrix metalloproteinase-recognized cleavage site is selected from the group consisting of GPLGMLSQ (SEQ ID NO:2) and GPLGLWAQ (SEQ ID NO:3).

Paragraph beginning at line 30 of page 6 has been amended as follows:

In one embodiment, the cell overexpresses a plasminogen activator or a plasminogen activator receptor. In another embodiment, the plasminogen activator is selected from the group consisting of t-PA (tissue-type plasminogen activator) and u-PA (urokinase-type plasminogen activator). In another embodiment, the plasminogen activator-recognized cleavage site is selected from the group consisting of PCPGRVVGG, PGSGRSA, PGSGKSA, and PQRGRSA (SEQ ID NOS:4-7, respectively).

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In one embodiment, the matrix metalloproteinase or a plasminogen activator-recognized cleavage site is selected from the group consisting of PCPGRVVGG, PGSGRSA, PGSGKSA, PQRGRSA, GPLGMLSQ and GPLGLWAQ (SEQ ID NOS:4-7, 2 and 3, respectively).

Paragraph beginning at line 33 of page 7 has been amended as follows:

Fig. 1. Generation of PA mutants can be specifically processed by MMPs. (A). Schematic representation of MMP substrate PA mutants. The furin cleavage site RKKR (SEQ ID NO:1) was replaced with gelatinase favorite substrate sequences GPLGMLSQ (SEQ ID NO:2) in PA-L1 and GPLGLWAQ (SEQ ID NO:3) in PA-L2. The arrows show the cleavage sites of furin or MMPs as indicated. (B). Cleavage of PA-L1 by MMP-2, MMP-9 and soluble form furin. As described in Materials and Methods, PA-L1 was incubated with MMP-2, MMP-9 and furin, respectively, aliquots were withdrawn at the time points indicated, and the samples were analyzed by western blotting with the rabbit polyclonal antibody against PA. (C). Cleavage of PA-L2 by MMP-2, MMP-9 and soluble form furin. PA-L2 was treated as in

Protective antigen proteins can be produced from nucleic acid constructs encoding mutants, in which the naturally occurring furin cleavage site has been replaced by an MMP or a plasminogen activator cleavage site. In addition, LF proteins, and LF and PA fusion proteins can also be expressed from nucleic acid constructs according to standard methodology. Those of skill in the art will recognize a wide variety of ways to introduce mutations into a nucleic acid encoding protective antigen or to construct a mutant protective antigen-encoding nucleic acid. Such methods are well known in the art (*see* Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2nd ed. 1989); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994)). In some embodiments, nucleic acids of the invention are generated using PCR (*see, e.g.*, Examples I and III). For example, using overlap PCR protective antigen encoding nucleic acids can be generated by substituting the nucleic acid subsequence that encodes the furin site with a nucleic acid subsequence that encodes a matrix metalloproteinase (MMP) site (*e.g.*, GPLGMLSQ and GPLGLWAQ; SEQ ID NOS:2 and 3) (*see, e.g.*, Example I). Similarly, an overlap PCR method can be used to construct the protective antigen proteins in which the furin site is replaced by a plasminogen activator cleavage site (*e.g.*, the uPA and tPA physiological substrate sequence PCPGRVVGG (SEQ ID NO:4), the uPA favorite sequence PGSGRSA (SEQ ID NO:5), the uPA favorite sequence PGSGKSA (SEQ ID NO:6), or the tPA favorite sequence PQRGRSA (SEQ ID NO:7)) (*see, e.g.*, Example III).

Paragraph beginning at line 26 of page 34 has been amended as follows:

Overlap PCR was used to construct the PA mutants with the furin site replaced by MMP substrate octapeptide GPLGMLSQ (SEQ ID NO:2) in PA-L1 and GPLGLWAQ (SEQ ID NO:3) in PA-L2. Wild type PA (WT-PA) expression plasmid pYS5 (Singh, Y., *et al.*, *J Biol Chem*, 264:19103-19107 (1989)) was used as template. We used 5' primer F (AAAGGAGAACGTATATGA (SEQ ID NO:8), underlined are SD sequence and start codon of PA) and the phosphorylated primer R1 (pTGAGTTCGAAGATTTTGTTTTAATTCTGG (SEQ ID NO:9), annealing to the sequence corresponding to P₁₅₄-S₁₆₃) to amplify the fragment N. We used the mutagenic phosphorylated primer H1 (pGGACCATTAGGAATGTGGAGTCAAAGTACAAGTGC TGGACCTACGGTTCCG (SEQ ID NO:10), encoding MMP substrate GPLGMLSQ (SEQ ID NO:2) and S₁₆₈-P₁₇₆) and reverse primer R2 ACGTTTATCTCTTATTAAAAT (SEQ ID NO:11), annealing to the sequence compassing I₅₈₉-R₅₉₅) to amplify the mutagenic fragment M1. We used a phosphorylated mutagenic primer H2 (pGGACCAT TAGGATTATGGGCACAAAGTACAAGTGCTGGACCTACGGTTCCG (SEQ ID NO:12), encoding MMP substrate GPLGLWAQ (SEQ ID NO:3) and S₁₆₈-P₁₇₆) to amplify mutagenic fragment M2. Then used primer F and R2 to amplify the ligation products of N and M1, N and M2, respectively, resulting in the mutagenic fragments L1 and L2, in which the coding sequence for furin site (RKKR₁₆₇; SEQ ID NO:1) were replaced by MMP substrate sequence GPLGMLSQ and GPLGLWAQ (SEQ ID NOS:2 and 3), respectively. The HindIII/PstI digests of L1 and L2, which included the mutation sites, were cloned between HindIII and PstI site of pYS5. The resulting expression plasmids were named pYS-PA-L1 and pYS-PA-L2, their expression products, the PA mutated proteins, were accordingly named PA-L1 and PA-L2.

Paragraph beginning at line 20 of page 39 has been amended as follows:

Crystal structure of PA showed that the furin cleavage site RKKR₁₆₇ is in the middle of a surface flexible, solvent exposed loop composed of aa 162 to 175 (Petosa, C., *et al.*, *Nature*, 385:833-838 (1997)). Cleavage in this loop by furin-like proteases is essential to toxicity. To construct PA mutants specifically processed by MMPs, especially MMP-2 and MMP-9, instead of furin, the furin site RKKR₁₆₇ (SEQ ID NO:1) was replaced by MMP-2 and MMP-9 favorite sequences, GPLGMLSQ and GPLGLWAQ (SEQ ID NOS:2 and 3), respectively, resulting in two PA mutants, PA-L1 and PA-L2 (Fig. 1a). These two MMP substrate octapeptides were designed based on the studies of Netzel-Arnett *et al* (Netzel-Arnett, S., *et al.*, *J Biol Chem*, 266:6747-6755 (1991); Netzel-Arnett, S., *et al.*, *Biochemistry*, 32:6427-6432 (1993)), in which the sequence specificity of human MMP-2, MMP-9, matrilysin, MMP-1 and MMP-8 had been examined by measuring the rate of hydrolysis of over 50 synthetic oligopeptides. These two octapeptides are favorite substrates of MMP-2 and MMP-9, but also overlap to other MMP species (Netzel-Arnett, S., *et al.*, *J Biol Chem*, 266:6747-6755 (1991); Netzel-Arnett, S., *et al.*, *Biochemistry*, 32:6427-6432 (1993)). They are also potential substrates for MT1-MMP (Will, H., *et al.*, *J Biol Chem*, 271:17119-17123 (1996)). PA-L1 and PA-L2 coding sequences were constructed by overlap PCR, cloned into *E. coli*-*Bacillus* shuttle vector pYS5, and efficiently expressed in non-virulent *Bacillus Anthracis* UM23C1-1. The expression products were secreted into the culture supernatants and reached to 20 to 50 mg/L. These two mutated PA proteins were roughly purified by ammonium sulfate precipitation, followed by mono Q chromatography. The purified mutated PA proteins PA-L1 and PA-L2 commiserated with WT-PA in SDS-PAGE, but migrated faster than WT-PA in native gel because of the four positively charged residues RKKR (SEQ ID NO:1) of the furin site were replaced into non-charged MMP octapeptides (data not shown).

Paragraph (Table 2) beginning at line 28 of page 43 has been amended as follows:

Table 2: u-TP and t-PA cleavage sites

Substrate sequence	<u>SEQ ID</u> <u>NO:</u>	u-PA Kcat/Km	t-PA Kcat/Km	a-PA:t-PA selectivity
PCPGRVVGG	<u>4</u>	0.88	0.29	3.0
PGSGRSA	<u>5</u>	1200	60	20
PGSGKSA	<u>6</u>	193	1.6	121
PQRGRSA	<u>7</u>	45	850	0.005

Paragraph beginning at line 16 of page 44 has been amended as follows:

A modified overlap PCR method was used to construct the mutated PA proteins in which the furin site is replaced by the uPA and tPA physiological substrate sequence PCPGRVVGG (SEQ ID NO:4) in PA-U1, uPA favorite sequences PGSGRSA (SEQ ID NO:5) and PGSGKSA (SEQ ID NO:6) in PA-U2 and PA-U3, respectively, tPA favorite sequence PQRGRSA (SEQ ID NO:7) in PA-U4. The PA expression plasmid pYS5 (Singh, Y., *et al.*, *J Biol Chem*, 264:19103-19107 (1989)) was used as template. A 5' primer F, AAAGGAGAACGTATATGA (SEQ ID NO:8) (Shine-Dalgarno and start codons are underlined), and the phosphorylated reverse primer R1, pTGGTGAGTTCGAAGATTTTGTTTTAATTCTGG (SEQ ID NO:13) (the first three nucleotides encodes P, the others anneal to the sequence corresponding to P₁₅₄--S₁₆₃), were used to amplify a fragment designated "N". A mutagenic phosphorylated primer H1, pTGTCAGGAAGAGTAGTTGGAGGAAGTACAAGTGCTGGACCTACGGTTCCAG (SEQ ID NO:14), encoding CPGRVVGG (SEQ ID NO:15) and S₁₆₈-P₁₇₆, and reverse primer R2, ACGTTTATCTCTTATTAAAAT (SEQ ID NO:11), annealing to the sequence encoding I₅₈₉-R₅₉₅, were used to amplify a mutagenic fragment "M1". A phosphorylated mutagenic primer H2, pGGAAGTGGAAGATCAGCAAGTACAAGTGCTGGACCTACGGTTCCAG (SEQ ID NO:16), encoding GSGRSA (SEQ ID NO:17) and S₁₆₈-P₁₇₆, and

reverse primer R2 were used to amplify a mutagenic fragment "M2". A phosphorylated mutagenic primer H3, pGGAAGTGGAATCAGCAAGTACAAGTGCTGGACCTACGGTTCCAG (SEQ ID NO:18), encoding GSGKSA (SEQ ID NO:19) and S₁₆₈-P₁₇₆, and reverse primer R2, were used to amplify a mutagenic fragment "M3". A phosphorylated mutagenic primer H4, pCAGAGAGGAAGATCAGCAAGTACAAGTGCTGGACCTACGGTTCCAG (SEQ ID NO:20), encoding QRGRSA (SEQ ID NO:21) and S₁₆₈-P₁₇₆, and reverse primer R2, were used to amplify a mutagenic fragment "M4". Primers F and R2 were used to amplify the ligated products of N + M1, N + M2, N + M3, and N + M4, respectively, resulting in the mutagenized fragments U1, U2, U3, and U4 in which the coding sequence for the furin site (RKKR₁₆₇; SEQ ID NO:1) is replaced by uPA or tPA substrate. The *Hind*III/*Pst*I digests of U1, U2, U3, and U4 were cloned between the *Hind*III and *Pst*I sites of pYS5. The resulting expression plasmids were named pYS-PA-U1, pYS-PA-U2, pYS-PA-U3, and pYS-PA-U4, and their expression products, the mutated PA proteins, were accordingly named PA-U1, PA-U2, PA-U3, and PA-U4. One expression plasmid encoded a mutant in which RKKR₁₆₇ (SEQ ID NO:1) is replaced by PGG, expected not to be cleaved by any protease. Its expression plasmid and expression product were named pYS-PA-U7 and PA-U7, respectively.

Paragraph beginning at line 6 of page 48 has been amended as follows:

The crystal structure of PA shows that the furin site, RKKR₁₆₇ (SEQ ID NO:1), is in a surface-exposed, flexible loop composed of aa 162 to 175 (Petosa, C., *et al.*, *Nature*, 385:833-838 (1997)). Cleavage in this loop by furin or furin-like proteases is essential to toxicity. Mutated PA proteins were constructed in which the furin-sensitive sequence RKKR₁₆₇ (SEQ ID NO:1) is replaced by uPA or tPA substrate sequences. In mutated PA protein PA-U1, PCPGRVVG (SEQ ID NO:4), a peptide from P5 to P4' in the physiological substrate plasminogen, was used to replace RKKR₁₆₇ (SEQ ID NO:1). In PA-U2, RKKR₁₆₇ (SEQ ID NO:1) was replaced by a peptide, PGSGRSA (SEQ ID NO:5), containing the consensus sequence SGRSA (SEQ ID NO:22) from P3 to P2',

which was recently identified as the minimized best substrate for uPA (Ke, S. H., *et al.*, *J. Biol. Chem.*, 272:20456-20462 (1997)). Because the peptide SGRSA (SEQ ID NO:22) is cleaved 1363-fold times more efficiently than a control peptide containing the physiological cleavage site present in plasminogen by uPA, and exhibits a uPA/tPA selectivity of 20 (Ke, S. H., *et al.*, *J. Biol. Chem.*, 272:20456-20462 (1997)), PA-U2 was expected to be a favorite substrate of uPA. uPA/tPA selectivity of the peptide SGRSA (SEQ ID NO:22) can be further enhanced by placement of lysine in the P1 position (Ke, S. H., *et al.*, *J. Biol. Chem.*, 272:20456-20462 (1997)), thus, the peptide PGSGKSA (SEQ ID NO:6), which exhibits a uPA/tPA selectivity of 121 (Ke, S. H., *et al.*, *J. Biol. Chem.*, 272:20456-20462 (1997)), was used to replace RKKR₁₆₇ (SEQ ID NO:1) to construct a mutated PA protein, PA-U3, with even higher uPA selective activity than PA-U2. The investigation showed P3 and P4 residues were the primary determinants of the ability of a substrate to discriminate between tPA and uPA, and mutation of both P4 glycine and P3 serine of the most labile uPA substrate (GSGRSA; SEQ ID NO:17) to glutamine and arginine, respectively, decreased the uPA/tPA selectivity by a factor of 1200 and actually converted the peptide into a tPA-selective substrate (Ke, S. H., *et al.*, *J. Biol. Chem.*, 272:20456-20462 (1997)). Based on this study, a mutated PA protein, PA-U4, was constructed. PA-U4 is expected to be a tPA favorite substrate, in which the peptide PQRGRSA (SEQ ID NO:7) was used to replace RKKR₁₆₇ (SEQ ID NO:1). A mutated PA protein PA-U7, was also constructed in which RKKR₁₆₇ (SEQ ID NO:1) was replaced by random sequence PGG, expected not to be cleaved by any known proteases, was used a control protein in this study. The designations of the mutated PA proteins along with the expected properties were summarized in Table 3.

Paragraph (Table 3) beginning at line 1 of page 55 has been amended as follows:

Table 3. PA proteins generated in this study

Designation	Sequence at the “furin loop”		SEQ ID	K _{cat} /K _m ¹		uPA:tPA	Protease expect to cleave
			NO:	uPA	tPA	selectivity ¹	
PA	NS RKKR↑	STSAGPTV	<u>23</u>				Furin
PA-U1	NSPCPGR↑	VVGGSTSAGPTV	<u>24</u>	0.88	0.29	3	uPA/tPA (weak
PA-U2	NSPGSGR↑	SA STSAGPTV	<u>25</u>	1200	60	20	uPA
PA-U3	NSPGSGK↑	SA STSAGPTV	<u>26</u>	193	1.6	121	uPA
PA-U4	NSPQRGR↑	SA STSAGPTV	<u>27</u>	7.3	670	0.005	tPA
PA-U7	NSPGG	STSAGPTV	<u>28</u>				None

[†]Data was cited from Ke, S. H., *et al.*, *J. Biol. Chem.*, 272:20456-20462 (1997) which was obtained from the studies on the peptides underlined in column 2.

In the Claims:

Claims 6, 7 and 24 have been amended as follows:

6. (Amended) The method of claim 1, wherein the matrix metalloproteinase-recognized cleavage site is selected from the group consisting of GPLGMLSQ (SEQ ID NO:2) and GPLGLWAQ (SEQ ID NO:3).

7. (Amended) The method of claim 1, wherein the plasminogen activator-recognized cleavage site is selected from the group consisting of PCPGRVVG (SEQ ID NO:4), PGSGRSA (SEQ ID NO:5), PGSGKSA (SEQ ID NO:6), and PQGRSA (SEQ ID NO:7).

24. (Amended) The method of claim 23, wherein the matrix metalloproteinase or a plasminogen activator-recognized cleavage site is selected from the group consisting PCPGRVVGG (SEQ ID NO:4), PGSGRSA (SEQ ID NO:5),

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PGSGKSA (SEQ ID NO:6), PQRGRSA (SEQ ID NO:7), GPLGMLSQ (SEQ ID NO:2) and GPLGLWAQ (SEQ ID NO:3).

PENDING CLAIMS WITH ENTRY OF THE AMENDMENT

1. (As filed) A method of targeting a compound to a cell over-expressing a matrix metalloproteinase, a plasminogen activator, or a plasminogen activator receptor, the method comprising the steps of:

(1) administering to the cell a mutant protective antigen protein comprising a matrix metalloproteinase or a plasminogen activator-recognized cleavage site in place of the native protective antigen furin-recognized cleavage site, wherein the mutant protective antigen is cleaved by a matrix metalloproteinase or a plasminogen activator; and

(ii) administering to the cell a compound comprising a lethal factor polypeptide comprising a protective antigen binding site; wherein the lethal factor polypeptide binds to cleaved protective antigen and is translocated into the cell, thereby delivering the compound to the cell.

2. (As filed) The method of claim 1, wherein the cell overexpresses a matrix metalloproteinase.

3. (As filed) The method of claim 2, wherein the matrix metalloproteinase is selected from the group consisting of MMP-2 (gelatinase A), MMP-9 (gelatinase B) and membrane-type1 MMP (MT1-MMP).

4. (As filed) The method of claim 1, wherein the cell overexpresses a plasminogen activator receptor.

5. (As filed) The method of claim 4, wherein the plasminogen activator is selected from the group consisting of t-PA and u-PA.

6. (Amended) The method of claim 1, wherein the matrix metalloproteinase-recognized cleavage site is selected from the group consisting of GPLGMLSQ (SEQ ID NO:2) and GPLGLWAQ (SEQ ID NO:3).

7. (Amended) The method of claim 1, wherein the plasminogen activator-recognized cleavage site is selected from the group consisting of PCPGRVVGG (SEQ ID NO:4), PGSGRSA (SEQ ID NO:5), PGSGKSA (SEQ ID NO:6), and PQRGRSA (SEQ ID NO:7).

8. (As filed) The method of claim 1, wherein the cell is a cancer cell.

9. (As filed) The method of claim 8, wherein the cancer is selected from the group consisting of lung cancer, breast cancer, bladder cancer, thyroid cancer, liver cancer, lung cancer, pleural cancer, pancreatic cancer, ovarian cancer, cervical cancer, colon cancer, fibrosarcoma, neuroblastoma, glioma, melanoma, monocytic leukemia, and myelogenous leukemia.

10. (As filed) The method of claim 1, wherein the cell is an inflammatory cell.

11. (As filed) The method of claim 1, wherein the lethal factor polypeptide is native lethal factor.

12. (As filed) The method of claim 1, wherein the compound is native lethal factor.

13. (As filed) The method of claim 1, wherein the lethal factor polypeptide is linked to a heterologous compound.

14. (As filed) The method of claim 13, wherein the compound is shiga toxin, A chain of diphtheria toxin, or *Pseudomonas* exotoxin A.

15. (As filed) The method of claim 13, wherein the compound is a detectable moiety.

16. (As filed) The method of claim 13, wherein the compound is a nucleic acid.

17. (As filed) The method of claim 13, wherein the compound is covalently linked to lethal factor via a chemical bond.

18. (As filed) The method of claim 13, wherein the heterologous compound is recombinantly linked to lethal factor.

19. (As filed) The method of claim 1, wherein the compound is a diagnostic or a therapeutic agent.

20. (As filed) The method of claim 1, wherein the cell is a human cell.

21. (As filed) The method of claim 1, wherein the mutant protective antigen protein is a fusion protein comprising a heterologous receptor binding domain.

22. (As filed) The method of claim 21, wherein the heterologous receptor binding domain is selected from the group consisting of a single chain antibody and a growth factor.

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23. (As filed) An isolated mutant protective antigen protein comprising a matrix metalloproteinase or a plasminogen activator-recognized cleavage site in place of the native protective antigen furin-recognized cleavage site, wherein the mutant protective antigen is cleaved by a matrix metalloproteinase or a plasminogen activator.

24. (Amended) The method of claim 23, wherein the matrix metalloproteinase or a plasminogen activator-recognized cleavage site is selected from the group consisting PCPGRVVGG (SEQ ID NO:4), PGSGRSA (SEQ ID NO:5), PGSGKSA (SEQ ID NO:6), PQRGRSA (SEQ ID NO:7), GPLGMLSQ (SEQ ID NO:2) and GPLGLWAQ (SEQ ID NO:3).